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TITLE: Dependency on Src-Family Kinases for Recurrence of Androgen-Independent Prostate Cancer

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14. ABSTRACT Prostate cancers that recur after so-called androgen-ablation therapy (“CR-CaP”) are typically more aggressive, more likely to spread to local lymph nodes and bones, and less likely to respond to second-tier treatments, and therefore, contribute to significantly decreased patient survival. We posit that enzymes called Src-family kinases (SFK) are required for the progression to CR-CaP, and thus, targeting these enzymes should prevent CR-CaP formation or suppress their growth. We have shown that inhibition of Src or Lyn by shRNA or drugs (Dasatinib or KX2-391) significantly decreases the frequency and time-to-formation of CR-CaP in the CWR22 model, and primary prostate cancer formation in the TRAMP mouse model. CR-CaP lesions that arose after Src knockdown exhibited parental levels of Src proteins, suggesting that Src is required for CR-CaP formation. Interestingly, the loss of Fyn enhanced the ability of TRAMP mice to form CaP lesions. Our pre-clinical studies identify role for certain SFK members, such as Src and Lyn, in CR-CaP disease. However, the therapeutic targeting of SFK in CR-CaP will likely require more specific inhibitors than Dasatinib so as not to also target SFK members such as Fyn, which might have CR-suppressing roles. This study, nonetheless, serves as a justification for the use of KX2-391 to treat or prevent CR-CaP					
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Introduction

We are studying the role of Src-family kinases (SFK) in promoting castration-recurrent prostate cancer (CR-CaP) using genetic and pharmacological approaches along with several animal models of CR-CaP. Our synergistic collaboration is based the expertise of the initiating PI (Gelman) in the molecular signaling of SFK in cancer progression, combined with the expertise of the partnering PIs in the CWR22 and TRAMP CR-CaP mouse models (Mohler and Smith, respectively), and in the role of neuroendocrine cells (NE) in the progression of CR-CaP (Smith).

Body

The following is a description of our synergistic research accomplishments in the past year in relation to the specific components of the original SOW (bolded).

Task 1. Produce CWR22 cells with tetracycline-regulated Src or Lyn-shRNA expression

Accomplishments to date-

We have procured and/or produced the necessary Src- and Lyn-specific shRNAs and cloned them into constitutive and inducible lentivirus vectors as described in the grant. These vectors express GFP as a marker of virus infection (driven by an IRES element in the virus construct), and in the tetracycline-inducible system, the lentivirus construct that expresses the tTR tet-i inducible transactivator, also expresses a DsRed cassette downstream of an IRES.

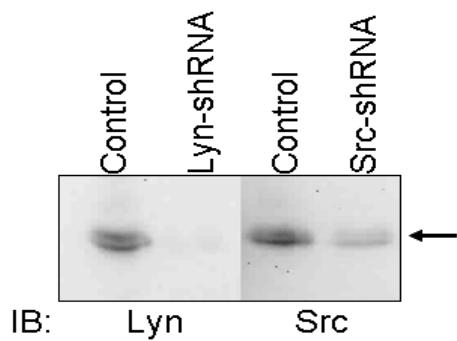


Fig. 1. IB of Lyn and Src in 293T cells infected at an MOI>1 with control, Src- and Lyn-shRNA lentiviruses.

These vectors were tested for their ability to knockdown human Src or Lyn protein levels. Thus, 293T cells were infected at multiplicities >1 GFP-forming virus/cell, and after 3-4 days of culture, the cell lysates were probed for Src or Lyn levels by immunoblotting (IB) with specific monoclonal antibodies (MAb). Fig. 1 shows that both shRNAs were able to knock down their respective targets roughly 8- to 10-fold compared to cells infected with control virus.

We then tested whether these viruses could efficiently infect primary cultures of androgen-dependent CWR22 tumor cells taken from male nude mice that were implanted with sustained release testosterone pellets. Thus, tumors around 250 mm^3 were removed, converted into single cell suspensions by incubation with collagenase, washed and the cell suspension infected with a titer of control or Src-shRNA lentiviruses that should yield roughly 90% infection. Fig. 2 shows that >90% of the CWR22 cells

showed the surrogate GFP marker for virus infection under these conditions, and indeed, this was even higher than the infectivity of 293T cells with the same virus stock.

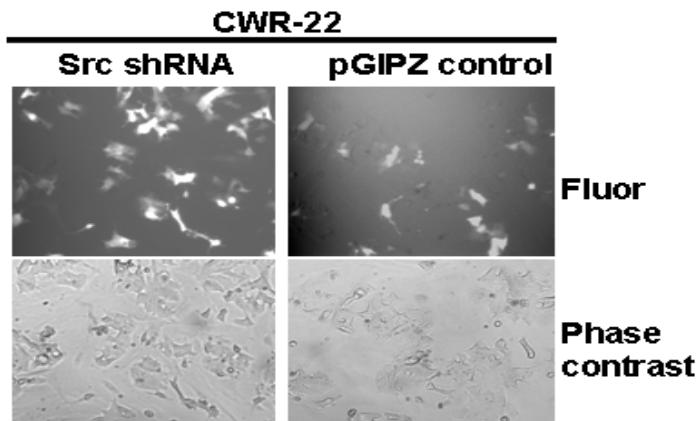


Fig. 3. Fluorescence (Fluor) and phase contrast microscopy of primary CWR22 and 293T cells infected with Src-shRNA or control pGIPZ lentivirus after three passages *in vitro*.

Src-shRNA/GFP lentivirus infection

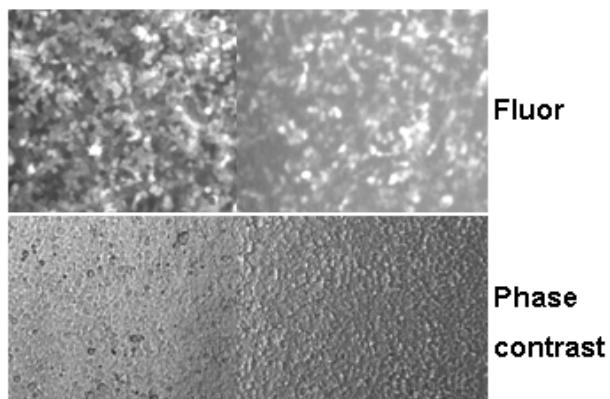


Fig. 2. Fluorescence (Fluor) and phase contrast microscopy of primary CWR22 and 293T cells infected with Src-shRNA lentivirus at an MOI=0.9.

The primary CWR22 tumor cells could be passaged at least three times until they began to senesce, but they retained their GFP expression during this period (roughly 2 weeks) as shown in Fig. 3. The ability to isolate single-cell populations of primary CWR22 cells, to efficiently transduce these cells, and then to reintroduce them into nude mice is not a trivial accomplishment. This success will allow us to continue our projected studies on the role of SFK and androgen receptor tyrosine phosphorylation in models of CR-CaP.

Table 1: Effect of Src shRNA on tumor occurrence	
Group (n = 10)	Recurrence
Control shRNA	4/10 (40%)
Src-shRNA	2/10 (20%)

The synergy in this Task is based on the production and testing of the lentiviruses by the Gelman lab, and the production of the CWR22 primary xenografts by the Mohler/Smith labs though the RPCI Mouse Tumor Model Resource.

Task 2. Inject nude mice with CWR22 Src- or Lyn-shRNA (or vector) cells

Accomplishments to date- We have infected our first set of primary cultures of CWR22 tumor cells harvested from tumors in testosterone-pelleted male nude mice (as described in Task 1) with control, Src- and Lyn-shRNA lentiviruses, and these cells have been reinjected s.c. at 10^6 cells + Matrigel into fresh testosterone-pelleted male nude mice. Mice were castrated when the primary tumor reached $150-250 \text{ mm}^3$, and the primary tumor regression was monitored. There was a small but statistically significant decrease in the rate of primary tumor regression in the group receiving the Src- vs. the control shRNA. However, the Src-shRNA group exhibited 50% fewer recurrent CaP lesions after 6 months compared to the control group (Table 1). Notably, all the recurrent tumors retained their strong lentivirus-encoded GFP expression (Fig. 4), indicating that there was sustained lentivirus expression 7-8 months after initial virus transduction into CWR22 cells. Whereas primary Src-shRNA tumors showed decreased levels of Src protein compared to primary tumors transduced with control virus (Fig. 5; compare lanes A-E with a-c), recurrent CR-CaP Src-shRNA tumors had control Src levels. This strongly suggests that recurrence requires sufficient Src activity.

The synergy in this Task is based on the production of the lentiviruses by the Gelman lab, and the production of the CWR22 primary xenografts by the Mohler/Smith labs though the RPCI Mouse Tumor Model Resource.

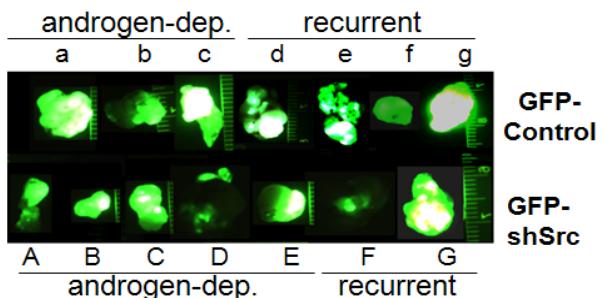


Fig. 4. Retention of lentivirus-encoded GFP in primary (androgen-dep.) and CR-CaP (recurrent) tumors from control or Src-shRNA transduced CWR22 cells.

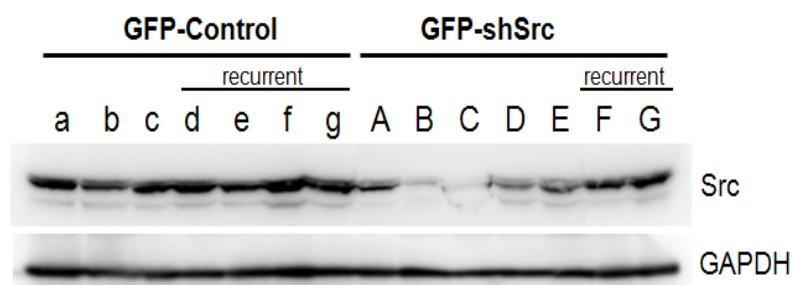


Fig. 5. Src-shRNA CR-CaP tumors exhibit parental Src protein levels found in primary or recurrent control tumors, compared to the knocked down Src levels in primary Src-shRNA tumors.

Task 3. Test whether KX2-391 (vs. vehicle or Dasatinib) can prevent recurrent Al-CaP or NE malignancy

Accomplishments to date- Castrated male C57BL/6 mice injected with TRAMP-C2H (Al-CaP) cells were treated with KX O1, Dasatinib or vehicle. We compared KX O1 dosing protocols and found that except for a lower general toxicity, there was no difference in the suppression of tumor growth by KX O1 dosed once vs. twice daily by oral gavage (Fig. 6). In contrast, constant dosing by adding the drug to the drinking water (assuming 11 ml water intake/20g mouse daily) was less effective at suppressing primary Al-tumor growth. This experiment is currently being repeated with a SID comparison to Dasatinib on primary Al-tumor growth. We started a metastasis experiment with mice whose primary C2H tumors were removed surgically, which were then treated with a 28-day regimen of KX O1, Dasatinib or vehicle SID. The mice are being

monitored for recurrent tumor growth at the primary site and for metastasis to draining lymph nodes. Whereas 6/10 mice receiving vehicle showed significant primary-site recurrences, only 1/10 mice in the KXO and Dasatinib groups showed recurrence, and these were small lesions. Data are forthcoming on the LN metastases.

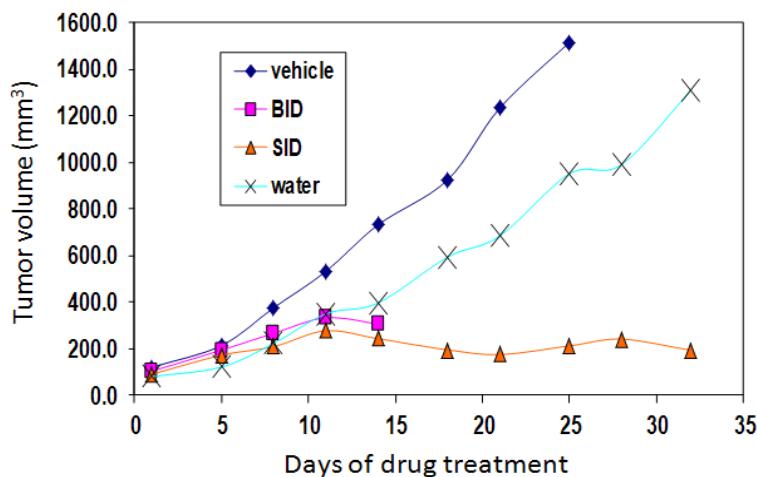


Fig. 6. Growth of preformed (250 mm^3) C2H TRAMP tumors in castrated male C57Bl/6 mice treated with vehicle alone or with KXO1 dosed in drinking water, or gavaged once (SID) or twice (BID) daily.

The effect of Dasatinib or KXO1 on CR-CaP generation was studied as shown in Fig. 7. The Src inhibitors were dosed orally once daily (*sid po*) for 28d starting 1d after castration, and then the mice were monitored for 6 months to identify the frequency of and time to recurrence. Our data (Table 2) indicate that Dasatinib or KXO1 inhibited CR-CaP formation by 50% and 60%, respectively, compared to vehicle alone. Moreover, both Src inhibitors caused a delay in time to recurrence (Fig. 8). Taken together, these data strengthen the notion that continued Src activity is required for CR-CaP generation, and thus, underlines the potential use of Dasatinib or KXO1 as treatments for clinical CR-CaP.

The synergy in this Task is based on the combined efforts by all three PIs' labs in regards to the mouse models and use of the Src-targeting drugs.

Task 4. Determine if AR^{Y534E} induces recurrent AI-CaP in Src- or Lyn-shRNA CWR22 cells

Accomplishments to date- Starting with an HA-tagged AR expression vector from Betty Wilson (UNC), we produced an HA-tagged AR^{Y534E} mutant expression vector. This has been verified by sequencing and is now undergoing testing for expression stability in 293T cells.

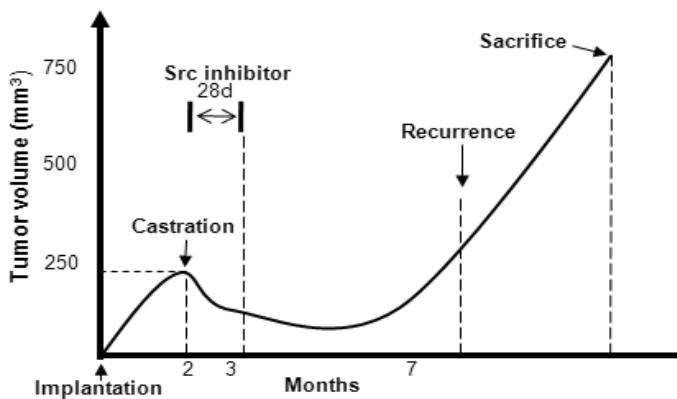
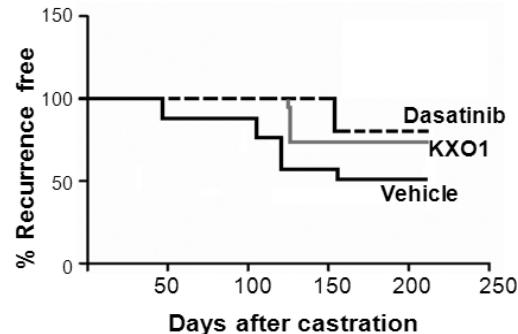


Fig. 7. Treatment regimen for Dasatinib or KXO1 (vs. vehicle) in the CWR22 xenograft system.

Table 1: Effect of KXO1 and Dasatinib on tumor occurrence

Group (n = 20)	Recurrence
Vehicle	10/20 (50%)
KXO1	4/20 (20%)
Dasatinib	5/20 (25%)



Task 5. Demonstrate increased NE proliferation in recurrent AI-CaP (CWR22) or NE (TRAMP) lesions

Accomplishments to date- CR-CaP tumors from control vs. Src-shRNA, or Dasatinib, KXO1 or vehicle treated CWR22-tumored mice were stained for the NE marker synaptophysin. Preliminary data indicate no change in the frequency of NE cells CR-CaP lesions. Given the parental levels of Src in the Src-shRNA CR-CaP lesions, it is likely that once CaP recurrence has been

Fig. 8. Time to recurrence in Dasatinib or KXO1 (vs. vehicle) treated CWR22 post-castration xenografts.

established (requiring Src), there is no difference between the population of NE cells surviving and proliferating in the CR-CaP.

Task 6. Produce Src-/- or Lyn-/- TRAMP mice, test for post-castration NE malignancy progression

Accomplishments to date- We have procured Src-/- mice (C57BL/6) from a pathogen-free facility (SUNY at Buffalo) and Fyn-/- and Lyn-/- frozen embryos from Jackson Labs. These mice have been crossed to TRAMP/TRAMP mice to generate males lacking Src, Lyn or Fyn and having one TRAMP allele. Fig. 9 shows that compared to mice expressing a single TRAMP allele (Probasin-SV40-Tag), the loss of Src or Lyn in the TRAMP+/- background

severely inhibited the onset of prostatic adenocarcinoma formation. In contrast, the loss of Fyn had an agonistic effect on the same TRAMP-induced tumor formation. These data suggest differing roles for members of the SFK family in promoting prostate cancer progression, and moreover, that in order to target SFK, specific inhibitors would be needed that selectively recognize Src and Lyn but not Fyn.

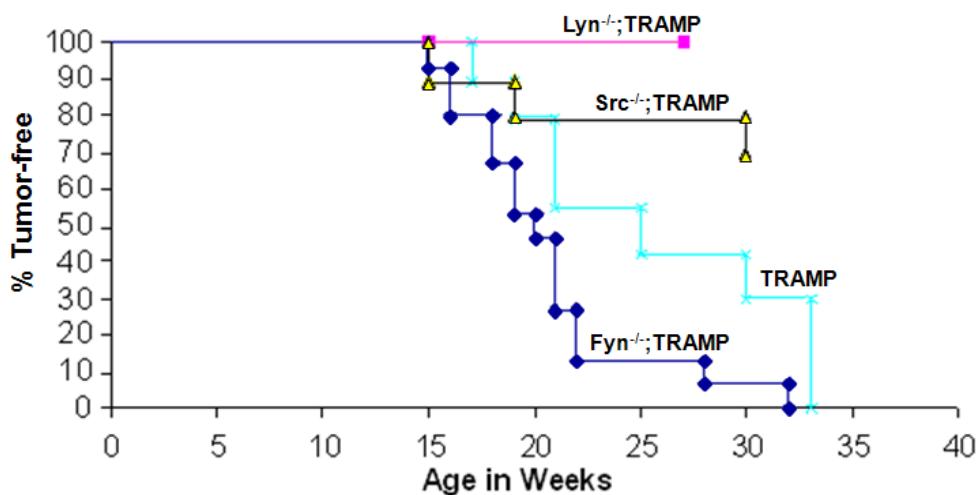


Fig. 9. Loss of Src or Lyn inhibited, and loss of Fyn agonized prostate cancer formation induced in the TRAMP+/- background.

Task 7. Transduce Src- or Lyn-null TRAMP early CaP cells with AR^{Y534E} or WT-AR, test for AI growth in castrated TRAMP mice

Accomplishments to date- Src-/-;TRAMP tumor cells have been isolated, cultured and transduced with AR^{Y534E} or WT-AR. These cells are being characterized for ectopic AR expression, and following this, will be injected into castrated male (syngeneic) mice.

Task 8. Analyze the role of SFK in NE-mediated AI-CaP growth human AD-CaP cell lines

Accomplishments to date- these experiments have not been started yet.

Task 9. Analyze the role of SFK in NE neuropeptide secretion

Accomplishments to date- these experiments have not been started yet.

Task 10. Analyze the role of SFK in NE proliferation and neuropeptide secretion *in vitro*

Accomplishments to date- these experiments have not been started yet.

Key Research Accomplishments

- production of Src- and Lyn-shRNA lentiviruses (constitutive and inducible expression)
- successful efficient transduction of primary androgen-dependent CWR22 tumor cells with shRNA-encoding lentiviruses
- demonstration of Src, Fyn and Lyn knockdown in human cells using the shRNA-encoding lentiviruses
- re-injection of testosterone pelleted SCID mice with transduced primary CWR22 cells
- demonstration of sustained lentivirus expression (based on GFP expression) for >5 months *in vivo* in castration-recurrent CWR tumors.
- demonstration that Src knockdown decreases CWR22 tumor recurrence *in vivo*.
- demonstration that KXO1 and Dasatinib can suppress AI-CaP recurrence at the primary

site in the TRAMP-C2H model.

-demonstration that KXO1 can suppress the growth of primary-site AI-CaP (C2H) and that once daily oral dosing is as potent as twice daily (with lower toxicity).

Reportable Outcomes

A manuscript, "Src controls castration-recurrence of CWR22 prostate cancer xenografts" has been prepared for submission for publication. This includes the Src-shRNA and SFK inhibitor data above. A second MS is being prepared for publication involving the role of SFK in TRAMP tumor formation.

Conclusion

The project is progressing at pace with no major obstacles. The synergistic component of the award has been critical to our success thus far and to our projected ability to proceed with the outstanding tasks. This project could not have been accomplished by each of the individual labs. Specifically, this project is progressing strictly because of the combining of the various expertise, such as the active use of the CWR22 and TRAMP models, the isolation and identification of NE cells, and the development and use of the shRNA-encoding lentiviruses. The PIs have also had scheduled meetings to strategize and to review data.

References

Appendices